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Diversity of GB Virus C/Hepatitis G Virus Isolates in Singapore: Predominance of Group 2a and the Asian Group 3 Variant

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The occurrence of GBV-C/HGV infection was studied in 160 individuals from two high-risk groups in Singapore. RT-PCR of the 5′-UTR detected GBV-C/HGV RNA in 3/73 (4.1%) of patients undergoing maintenance hemodialysis for chronic renal failure, and in 17/87 (19.5%) of patients coinfected with HCV who tested positive for HCV RNA. Phylogenetic analysis of 5′-UTR sequences from these 20 samples showed that the Asian or group 3 variant was found in 45% of the samples sequenced, thus confirming the high frequency of this variant in the region. Group 2a variants accounted for 50% of the samples with a complete absence of group 2b. Our analysis also provided strong bootstrap support for the subdivision of group 2 into subgroups 2a and 2b. This study shows that isolates belonging to all three main groups of GBV-C/HGV can be detected in Singapore, with the large majority belonging to groups 2a (50%) and 3 (45%). Only a single group 1-like sequence was detected within the 20 isolates. Of interest also is that all group 3 isolates were identified in Chinese patients while group 2a was found in both Chinese and Malay. ***J. Med. Virol. 58:145–153, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: GBV-C/HGV; 5′-UTR sequences; phylogenetic groups; Singapore; hepatitis; Asian

INTRODUCTION

Two novel hepatitis C-like flaviviruses were recently isolated from patients with chronic hepatitis. GB virus-C (GBV-C) was first reported in 1995 [Simons et al., 1995], followed by hepatitis G virus (HGV) [Linnen et al., 1996]. A comparison of the two viral genomes indicated that they shared significant sequence identity and that GBV-C and HGV were two different isolates of the same virus, hereafter referred to as GBV-C/HGV. The genomic organization of GBV-C/HGV is similar to that of HCV and other flaviviruses. The single open reading frame encodes structural and nonstructural proteins and is flanked by 5′ and 3′ untranslated regions.

Several studies have indicated that GBV-C/HGV is spread parenterally and sexually. This mode of transmission had prompted the need to study the virus's diversity and distribution [Alter et al., 1997a, 1997b]. Firstly, there is a significant coinfection rate with the hepatitis B and C viruses, ranging from 5%–14% for HBV [Nakatsuji et al., 1996; Guilera et al., 1998] and 7%–31% for HCV [Gonzalez-Perez et al., 1997; Guilera et al., 1998]. This suggested shared modes of transmission. Subsequently, it was shown that patients with known parenteral exposures shared high levels of GBV-C/HGV viremia not shown by the general population. These groups at high risk for GBV-C/HGV viremia included parenteral drug addicts, patients on hemodialysis, known recipients of blood transfusions, and hemophiliacs. It was also shown that GBV-C/HGV could be isolated from the semen of men known to be viremic with GBV-C/HGV [Semprini et al., 1998]. Others have described possible interspousal spread [Kao et al., 1997b] and vertical transmission from mother to child [Zanetti et al., 1998], in both instances at apparently more efficient rates than the transmission of HCV. In this article, we focus on GBV-C/HGV viremia in two high-risk groups in Singapore, namely, the chronic recipients of hemodialysis and patients viremic with HCV. As sequence information for the group 3 Asian variants is limited and thus far mainly from Japanese studies, it is important to determine the prevalence and polymorphism of such variants in other parts of Asia.

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MATERIALS AND METHODS

Test Subjects

Sera was obtained from 73 patients undergoing regular maintenance hemodialysis at the Renal Dialysis Center of the National University Hospital. Six of these patients are hepatitis B carriers as determined by positive serum HBsAg, of which three were also HBV DNA-positive. Another 16 patients were positive for HCV IgG by EIA. None of the patients were simultaneously infected by both HBV and HCV viruses. The remaining 51 patients were negative for both HBV and HCV. A second group of high-risk individuals were selected from patients who had been tested positive for HCV RNA by RT-PCR at the Department of Microbiology at the National University of Singapore.

Extraction of GBV-C/HGV RNA From Sera

Viral RNA from 200 μL of sera was extracted using the High-Pure Viral RNA spin column kit (Boehringer Mannheim, Indianapolis, IN). In brief, the sera was mixed with 400 μL of lysing agent containing guanidinium isothiocyanate. This mixture was then placed in the upper chamber of a spin-column and centrifuged at 10,000 g for 15 seconds. This was followed by two ethanol wash steps. The RNA was subsequently eluted from the glass fibers in the spin column with 50 μL of nuclease-free water, and used immediately for subsequent cDNA synthesis.

cDNA Synthesis

Fifty μL of the serum precipitate material was heated to 70°C in the presence of random nucleotide hexamers. The mixture was plunged into ice and subsequently reverse-transcribed with murine Moloney virus reverse transcriptase (Superscript II, Gibco-BRL, Bethesda, MD) at 40°C for 60 min. cDNA was either immediately used for PCR or stored at –20°C.

PCR Amplification of GBV-C/HGV 5′-UTR and Detection of PCR Products

Eighty-three GBV-C/HGV sequences obtained from GenBank were aligned using the program CLUSTAL W. These sequences included those with GenBank accession numbers AB003673–003679, D84533–84543, D87249–87254, HDU76892–76894, AB008335, AB008342, AF031827–031829, D87708–87714, U89148–89158, U44402, U45966, and D83501–83503. PCR primers for amplification of the GBV-C/HGV 5′-UTR were derived from the extreme 5′-UTR and the E1 portions of the genome. Additional primers were identified from the literature [Muerhoff et al., 1996; Wu et al 1997].

For the initial detection of GBV-C/HGV RNA, 2.5 μL of cDNA was amplified in a reaction mix of final volume 25 μL, containing 1.5-mM Mg^{2+} and 100-mM dNTP (outer forward primer F42: 5′-TGCAAGCCCCAGAAACCGAC-3′; reverse: R361: 5′-GAGACATTGAAGGGCGACGTGG-3′; 2.5 μL of the first-round PCR product was subsequently used as template for second-round nested reaction forward-nested primer F163: 5′-CTTGGTAGCCACTATAGGTG-3′; reverse-nested 5gr4: 5′-GCGACGTGGACCGTACGTGGGCG-3′). For both PCR steps, cycling conditions were as follows: an initial hot start of 96°C for 5 min; followed by denaturation at 96°C for 45 sec, annealing at 60°C for 1 min and strand elongation at 72°C for 2 min for a total of 35 cycles, and a final elongation phase of 72°C for 10 min. Fifteen μL of second-round PCR product was visualized in an ethidium bromide-stained 4% agarose gel.

To avoid scoring false positives, samples were only considered positive for GBV-C/HGV when reproducible results were obtained from at least two separate runs on different days. Strict procedures were also observed to avoid cross-contamination.

Due to limited information available for the Asian or group 3 variants, different primers were used for amplifying the GBV-C/HGV 5′-UTR in order to obtain sequence data. Amplification was always first attempted with the following primers: outer sense F4, 5′-GACGTGGGGGGGTTGATC-3′; antisense R653, 5′-TTGTGAGGAAATATTGCCC-3′; inner sense F31, 5′-CGGCACTGGGTGCAAGCCCCAG-3′; antisense R604, 5′-CAGAATGGCCCCGGCCTC-3′. Cycling conditions are as follows: hot start of 96°C for 5 min, followed by denaturation at 96°C for 45 sec, annealing at 63°C for 1 min, and strand elongation at 72°C for 2 min, for a total of 35 cycles, and a final elongation stage of 72°C for 10 min. If this primer set failed to amplify the GBV-C/HGV 5′-UTR, various combinations of primers were used to derive overlapping fragments of as much of the 5′-UTR as possible. In all, five different sense primers, F4, F31, S1 (5′-GGCACTGGGTGCAAGCCCCAG-3′), F42, and F163, as well as six antisense primers, R653, R604, 4R (5′-CGGAGCTGGGTGGCCCCATGC-3′), R477 (5′-GGTGGCCCCATGCATTTC-3′), R361, and 5gr4, were used. Some or all of these, with the addition of R165 (5′-CACCTATAGTGGCTACCA-3′), were used as sequencing primers.

Sequencing and Analysis of GBV-C/HGV 5′-UTR

One hundred μL of nested PCR product was run in a 2% agarose gel. The appropriate band was excised and processed using the Qiaquick gel extraction kit (Qiagen, Santa Clarita, CA). Purified DNA was eluted with 30–50 μL of water and used immediately for sequencing, or stored at –20°C.

A total of 8.8 μL of purified PCR product was subsequently used for sequencing, using d-Rhodamine labeled fluorescent deoxynucleotides in an ABI PRISM 310 automated sequencer (Perkin-Elmer, Norwalk, CT). In order to minimize sequence errors introduced during reverse transcription and PCR amplification, all GBV-C/HGV cDNAs were derived at least twice from each sera, and the 5′-UTR was sequenced at least twice in both directions.

Sequence Analysis

In order to analyze the sequences obtained in this study, we evaluated them against published GBV-C/

HGV sequences obtained from GenBank. These included the GBV-C prototype (U36380 from Leary et al. [1996]), the HGV prototype PNF 216I and HGV-JC (U44402 and U45966 from Linnen et al. [1996]), as well as others; details are summarized in Table I. The large number of sequences were used in order to strengthen the bootstrap support for the groupings obtained from phylogenetic analysis.

Phylogenetic analyses were carried out using the PHYLIP package version 3.5c [Felsenstein, 1993], with the sequences first aligned using the multiple sequence alignment option in the CLUSTAL W program version 1.7 [Higgins and Sharp, 1988; Thompson et al., 1994]. These aligned sequences were subject to bootstrap resampling (1,000 samplings) using the SEQBOOT module in PHYLIP. Thereafter, the bootstrapped data were subjected to two different methods of analysis. In the first instance, the input order of sequences in each of the 1,000 bootstrapped samples was JUMBLED 10 times, and DNAPARS was used to find the most parsimonious tree for each sample. A majority-rule consensus tree was constructed with CONSENSE. Activating the JUMBLE option reduces the effect of sequence input order on the output branching order of the isolates, particularly when the isolates are expected to be greatly similar. The other method of analysis involved calculating the Kimura two-parameter pairwise distances between isolates in each of the bootstrap samplings with DNADIST. Based on these distances, phylogenetic trees were constructed using the neighbor-joining method of Saitou and Nei [1987], from which a consensus tree was derived with CONSENSE. The consensus treefiles produced by CONSENSE were visualized using TREEVIEW [Page, 1996].

RESULTS

Detection of GBV-C/HGV in Two High-Risk Groups

Primers to the 5′-UTR region was used in this study as it has been shown to be more sensitive and have a higher detection rate than primers to other regions of the GBV-C/HGV genome [Cantaloube et al., 1996; Kao et al., 1997a]. Moreover, in contrast with studies of the 5′-UTR, phylogenetic analysis of small fragments of the coding region do not reproduce groupings that correlate with geographic origin of the isolate [Muerhoff et al., 1996; Pickering et al., 1997; Viazov et al., 1997]. The results using 5′-UTR primers show that in the patients group who are on maintenance hemodialysis for chronic renal failure, 3/73 (4.1%) tested positive for GBV-C/HGV RNA. One patient, SG26, was coinfected with both HBV DNA and GBV-C/HGV RNA (6/73 of this group were HBsAg-positive); while one other patient, SG57, was tested positive for HCV RNA and GBV-C/HGV RNA (16/73 were anti-HCV–positive). Hence the occurrence of coinfectivity in the hemodialysis group is not high. In the second group of patients who are HCV carriers and their sera tested positive for HCV RNA, 17/87 (19.5%) were found to be GBV-C/HGV RNA-positive.

Support for Major Groups and Subgroups

Among the 20 GBV-C/HGV isolates that were sequenced in this study, 15 isolates were sequenced up to or beyond the ATG start codon and 516-bp information was used in phylogenetic analysis along with 57 other published sequences (Fig. 1).

Five isolates—SG3279, SG3283, SG3312, SG3315, and SG3399—did not amplify with any of the five E1-based primers. Therefore, relatively truncated PCR products were obtained that did not extend to the ATG initiation codon. An analysis of all the 20 local isolates using 410-bp information produced results that are in good agreement with the 516-bp analysis (data not shown).

Bootstrap values obtained for the consensus tree after subjecting 1,000 bootstrapped resamplings of the aligned sequences through DNADIST (Kimura two-parameter method) followed by NEIGHBOR were compared with results obtained from the parsimony module, DNAPARS (Table II). In this study, the bootstrap proportions in support of the three major groupings range between 99.1%–99.9% for group 1, 77.3%–100% for group 2, and 99.2%–100% for group 3. The putative subdivision of group 1 into 1a and 1b occurred in 49.0%–66.8% and 65.1%–80.3% of the bootstrapped samples, respectively. The bootstrap proportions observed for group 2a was 26.3%–79.7%, and that for 2b 99.8%–100.0%. The general trend observed was that the parsimony method yielded higher bootstrap proportions than the distance method of analysis. The exception was the analysis for the putative subgroup 1a, where the distance method yielded slightly higher bootstrap values, regardless of sequence length. Comparing the two parsimony runs, the analysis of 72 sequences of 516 nucleotides each provided slightly higher bootstrap values than the analysis of the 79 sequences of 410 nucleotides each.

Assignment of Reference Isolates to Their Groups

With one exception, the reference isolates obtained from GenBank were assigned correctly to their previously determined groups in all four analyses. The unusual Chinese isolate HGVC964 (U75356) was placed in subgroup 2a where it was paired with HGV-JC (U45966) with a bootstrap proportion of 100% by the parsimony method. However, it was placed as a distinct branch from the rest of group 2 in 67.8% of bootstraps by the distance method of analysis.

Assignment of Singaporean Isolates to Their Groups

The majority of the Singaporean isolates in this study were distributed among groups 2 and 3. Nine of 20 isolates (45%) grouped with the reference isolates in group 3. These were SG72, SG3279, SG3329, SG3332, SG3334, SG3399, SG3491, SG3403, and SG3410. Ten of 20 Singaporean isolates (SG26, SG2813, SG3283, SG3312, SG3315, SG3315, SG3322, SG3327, SG3362,

TABLE I. Characteristics of the GBV-C/HGV Isolates Used in the Phylogenetic Analysis

Isolate	Accession number	Country/race	Group	Diagnosis	Reference
Isolate 23	U59540	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 24	U59541	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 25	U59542	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 26	U59543	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 27	U59544	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 28	U59545	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 29	U59546	Ghana	1	Unknown	Muerhoff et al. (1996)
GBV-C	U36380	Ghana	1	Unknown	Leary et al. (1995)
Isolate 31	U59547	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 32	U59548	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 33	U59549	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 34	U59550	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 35	U59551	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 36	U59552	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 37	U59553	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 38	U59554	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 39	U59555	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 40	U59556	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 41	U59557	Ghana	1	Unknown	Muerhoff et al. (1996)
Isolate 42	U59558	Ghana	1	Unknown	Muerhoff et al. (1996)
SG0057	AF078799	Singapore Malay	1	Hemodialysis	This study
SG0026	AF078047	Singapore Chinese	2a	Hemodialysis; anti-HCV$^+$	This study
SG2813	AF078050	Singapore Chinese	2a	Hemodialysis; anti-HCV$^+$	This study
SG3283	AF078052	Singapore Chinese	2a	Hemodialysis; anti-HCV$^+$	This study
SG3312	AF078053	Singapore Malay	2a	Hemodialysis; anti-HCV$^+$	This study
SG3315	AF078799	Singapore Malay	2a	Hemodialysis; anti-HCV$^+$	This study
SG3316	AF078054	Singapore Chinese	2a	Hemodialysis; anti-HCV$^+$	This study
SG3322	AF078055	Singapore Chinese	2a	Hemodialysis; anti-HCV$^+$	This study
SG3327	AF078056	Singapore Chinese	2a	Hemodialysis; anti HCV$^+$	This study
SG3362	AF078060	Singapore Malay	2a	Hemodialysis; anti-HCV$^+$	This study
SG3419	AF078065	Singapore Chinese	2a	Hemodialysis; anti-HCV$^+$	This study
Isolate 01	U59518	United States	2a	Unknown	Muerhoff et al. (1996)
Isolate 02	U59519	United States	2a	Unknown	Muerhoff et al. (1996)
Isolate 03	U59520	Greece	2a	Unknown	Muerhoff et al. (1996)
Isolate 04	U59521	Italy	2a	Unknown	Muerhoff et al. (1996)
Isolate 05	U59522	United States	2a	Unknown	Muerhoff et al. (1996)
Isolate 06	U59523	United States	2a	Unknown	Muerhoff et al. (1996)
Isolate 07	U59524	United States	2a	Unknown	Muerhoff et al. (1996)
Isolate 08	U59525	United States	2a	Unknown	Muerhoff et al. (1996)
Isolate 09	U59526	United States	2a	Unknown	Muerhoff et al. (1996)
Isolate 10	U59527	United States	2a	Unknown	Muerhoff et al. (1996)
Isolate 11	U59528	United States	2a	Unknown	Muerhoff et al. (1996)
GT 110	D90600	Japan	2a	Blood donor	Okamoto et al. (1997)
T55875	AF031827	United States	2a	Blood donor	Bukh et al. (1998)
HGV-1517	AF031828	United States	2a	Chimp 1517	Bukh et al. (1998)
HGV-1539	AF031829	United States	2a	As above	Bukh et al. (1998)
PNF 216 I	U44402	United States	2a	Chronic hepatitis	Linnen et al. (1996)
HGV-JC	U45966	United States	2a	Chronic hepatitis	Linnen et al. (1996)
HGV-Iw	D87255	Japan	2a	Hepatitis	Shao et al. (1996)
GBV-C(EA)	U63715	East Africa	2b	Acute hepatitis	Erker et al. (1996)
Isolate 12	U59529	Greece	2b	Unknown	Muerhoff et al. (1996)
Isolate 13	U59530	Greece	2b	Unknown	Muerhoff et al. (1996)
Isolate 14	U59531	United States	2b	Unknown	Muerhoff et al. (1996)
Isolate 15	U59532	United States	2b	Unknown	Muerhoff et al. (1996)
Isolate 16	U59533	East Africa	2b	Unknown	Muerhoff et al. (1996)
Isolate 17	U59534	Greece	2b	Unknown	Muerhoff et al. (1996)
Isolate 18	U59535	United States	2b	Unknown	Muerhoff et al. (1996)
Isolate 19	U59536	United States	2b	Unknown	Muerhoff et al. (1996)
Isolate 20	U59537	United States	2b	Unknown	Muerhoff et al. (1996)
SG0072	AF078049	Singapore Chinese	3	Hemodialysis	This study
SG3279	AF078051	Singapore Chinese	3	HCV RNA$^+$	This study
SG3322	AF078055	Singapore Chinese	3	HCV RNA$^+$	This study
SG3329	AF078057	Singapore Chinese	3	HCV RNA$^+$	This study
SG3334	AF078059	Singapore Chinese	3	HCV RNA$^+$	This study
SG3399	AF078061	Singapore Chinese	3	HCV RNA$^+$	This study
SG3401	AF078062	Singapore Chinese	3	HCV RNA$^+$	This study
SG3403	AF078063	Singapore Chinese	3	HCV RNA$^+$	This study

TABLE I. Continued

Isolate	Accession number	Country/race	Group	Diagnosis	Reference
SG3410	AF078064	Singapore Chinese	3	HCV RNA$^+$	This study
K3732	AB008335	Japan	3	Unknown	Katayama et al. (1998)
HGV-IM71	AB008342	Japan	3	Unknown	Data not shown
K606	D87708	Japan	3	Suspected viral hepatitis	Katayama et al. (1997)
K1737	D87709	Japan	3	Suspected viral hepatitis	Katayama et al. (1997)
K1741	D87710	Japan	3	Suspected viral hepatitis	Katayama et al. (1997)
K1789	D87711	Japan	3	Suspected viral hepatitis	Katayama et al. (1997)
K1916	D87712	Japan	3	Suspected viral hepatitis	Katayama et al. (1997)
K2141	D87713	Japan	3	Suspected viral hepatitis	Katayama et al. (1997)
K1668	D87714	Japan	3	Suspected viral hepatitis	Katayama et al. (1997)
Isolate 22	U59539	Japan	3	Unknown	Muerhoff et al. (1996)
HGVC964	U75356	South China	?	Unknown	Zhou et al. (1996)

and SG3419) segregated with other isolates in group 2a. None of our samples were grouped with 2b isolates. Group 2a isolates were found among Chinese as well as Malay Singaporeans. All group 3 isolates were from Chinese patients. Isolate SG57 isolated from a Malay patient was peculiar, as it was the only isolates not to segregate with the others in group 2a or 3. Furthermore, it was distinct from the reference isolates from group 1 that were included as a comparison in this study. SG57 branched off from the rest of group 1 consistently in all our analyses with a bootstrap proportion of 61.5%–77.0%, with the higher bootstrap proportions being observed in analyses using parsimony methods.

DISCUSSION

This is the first detailed survey and analysis of GBV-C/HGV frequency and variant groups in Singapore. This study was undertaken as it is apparent that there exists a correlation between the variants of this virus and their geographic origin. Group 1 isolates have been identified mainly in Africa, group 2 isolates have been associated with America and Europe, and group 3 isolates are Asian. Sequence data for this last variant group are scarce and it is necessary to validate the polymorphism and distribution of this group. A better understanding of the polymorphic variants and their global distribution will help to elucidate the importance of this virus [Miyakawa et al., 1997]

In addition to the three main established groups, there has been recent evidence suggesting that GBV-C/HGV may be even more heterogeneous. In particular, more Asian groups may remain to be uncovered. A study that included in its analysis HGVC964 (U75356), an isolate from southern China, suggested that it was a novel isolate that could be a prototype of group 4 [Smith et al., 1997]. However, this claim was based on an analysis of only 132 nucleotides (nucleotide –366 to –235) from the GBV-C/HGV 5′-UTR. Another analysis [Muerhoff et al., 1997] that included 374 nucleotides from the 5′-UTR of this isolate grouped it with other isolates in group 2a, but an examination of the E2, NS3, and NS5b sequence segregated it together with other group 3 isolates. Furthermore, a recent survey for the anti-E2 antibody directed against GBV-C/HGV E2 glycoprotein [Pilot-Matias et al., 1996; Dille et al., 1997] noted that sera from Asians had notably lower rates of seropositivity than sera from elsewhere in the world [Ross et al., 1998]. One explanation for this discrepancy might be the presence of Asian strains with unusual envelope proteins that were not being detected by the commercial antibody kits used in this study. However, an analysis of a 310-nucleotide stretch of the E2 sequence yielded a single distribution of evolutionary distances, and the correlation of phylogenetic groupings with geographic origin seen with analysis of the 5′-UTR was not reproduced [Muerhoff et al., 1997]. This suggests that variation within the E2 may not be significant and does not account for the low rate of anti-E2 seropositivity. However, it does not rule out the effects of point mutations that might not be numerous enough to distinguish between the isolates in a phylogenetic sequence analysis, but might affect critical epitope changes in the E2 product that influence the immune response against it.

The occurrence of GBV-C/HGV infection in patients undergoing hemodialysis has been reported to range from 3.5% [Okuda et al., 1997] to as high as 34.6% in a Turkish study [Gunaydin et al., 1997]. The occurrence of GBV-C/HGV RNA in 4.1% of our study population of hemodialysis patients is on the low side of this range. In contrast, the GBV-C/HGV coinfection rate of 19.5% in our cohort of HCV-positive patients is in the middle of the 7%–31% range that has been reported [Gonzalez-Perez et al., 1997; Guilera et al., 1998]. The occurrence of GBV-C/HGV viremia is in this patient group. There is evidence to suggest that detection in sera of antibody directed against the GBV-C/HGV E2 glycoprotein might be correlated with clearance of previous viral infection [Hassoba et al., 1997; Tacke et al., 1997a; 1997b], thus increasing the coinfection rate. True prevalence of GBV-C/HGV infection would then be a sum of both GBV-C/HGV RNA as well as anti-E2 rates.

As has been previously pointed out [Muerhoff et al., 1996); Kondo et al., 1997], there appears to be differential evolutionary pressures exerted 5′ and 3′ of the putative polyprotein ATG start codon of GBV-C/HGV. The polyprotein coding sequences are heavily conserved, whereas the 5′-UTR exhibits a greater degree

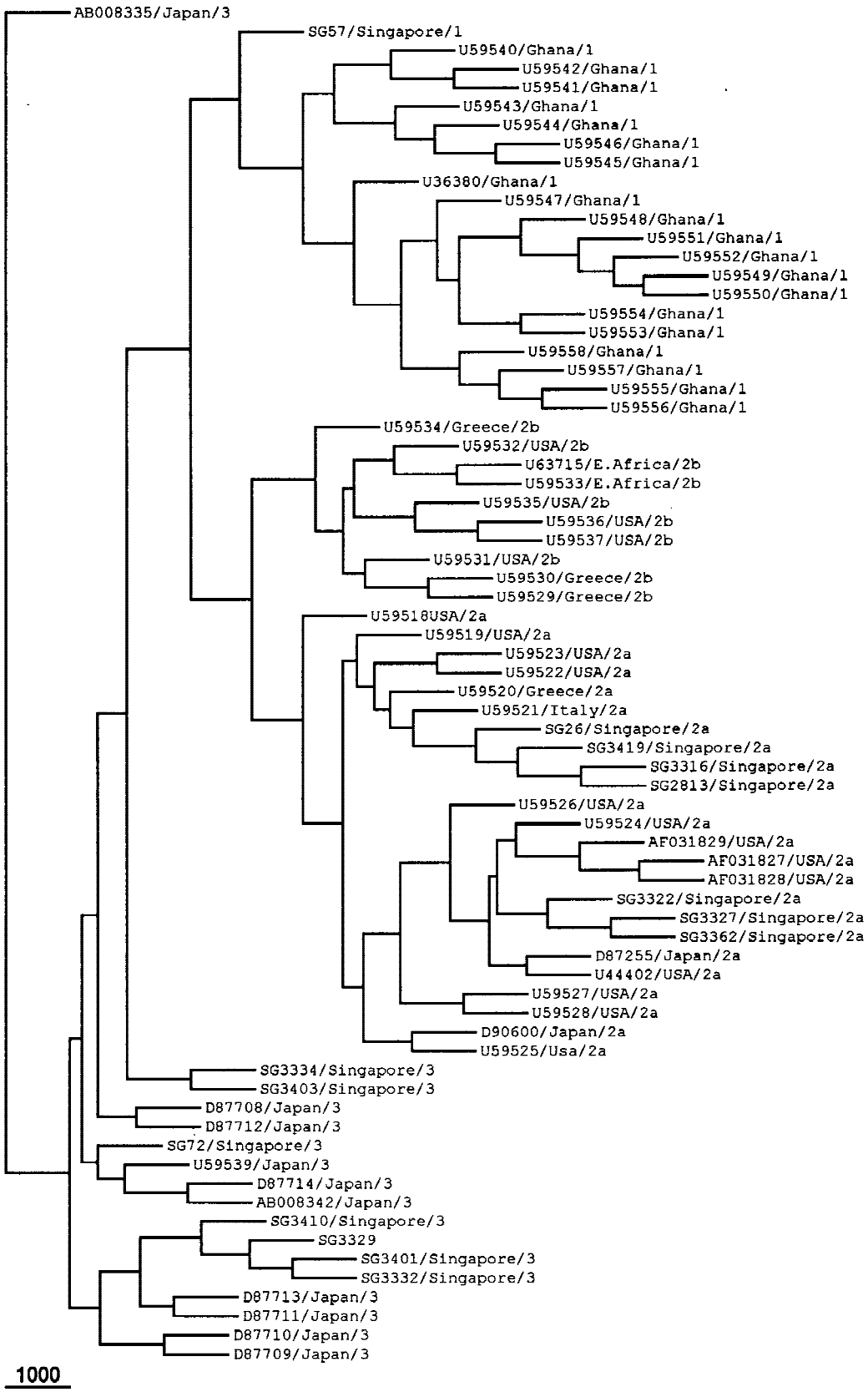

Fig. 1. Phylogenetic analysis of 72 aligned sequences (516 bp each) employing the parsimony method. Previously published sequences are indicated by their GenBank accession number and the newly identified Singapore isolates by their isolate names. The sequences segregated into four major groups (1, 2a, 2b, and 3) as indicated by their respective number at the end of each isolate, along with its country of origin.

TABLE II. Phylogenetic Analysis of 516-bp vs. 410-bp 5′-UTR Sequence[a]

Region analyzed	Number of bases	Number of sequences	Method of analysis	Bootstrap proportions *P*/%								
				1	1a	1b	2	2a	2b	3	SG57	U75356
−500 to −1	516	72	DNAPARS	99.9	49.0	80.3	95.9	79.7	99.8	99.9	77.0	
−500 to −1	516	72	DNADIST, NJ	99.6	56.9	77.4	100.0	59.0	99.8	100.0	73.4	
−500 to −103	410	77	DNAPARS	99.4	58.1	65.1	98.4	70.4	100.0	99.2	61.5	100.0
−500 to −103	410	77	DNADIST, NJ	99.1	66.8	65.2	77.9	26.3	99.9	99.9	73.0	67.8

[a]Bootstrap proportions obtained for GBV-C/HGV groups, subgroups, and unusual isolates from the phylogenetic analysis of either a 516 or 410 nucleotide alignment. The numbering is based on the GBV-C prototype isolate U36380 [Leary et al., 1996], and the nucleotides are given increasingly negative numbers 3′ of the putative ATG initiation codon. The number of bases analyzed appears larger than what is suggested by the numbering of region analyzed because of insertions in any or all of the other sequences in the alignment, which increased the number of bases in the alignment, relative to the 5′-UTR of the GBV-C prototype. DNAPARS indicates the parsimony module in PHYLIP. DNADIST is the distance matrix module in PHYLIP, and the Kimura two-parameter distance was computed. NJ refers to NEIGHBOR, the neighbor-joining module in PHYLIP. The bootstrap proportions indicate the percentage with which the group of interest occurred among the 1,000 bootstrapped resamplings.

of variation. Moreover, analysis of a 374-nucleotide region of the 5′-UTR was sufficient to discriminate the three main groupings of GBV-C/HGV that correlated with geographic origin [Muerhoff et al., 1997]. Analysis of a much longer portion of the coding sequence, a concatenate of the E2, NS3, and NS5b regions, was necessary before a similar relationship could be reproduced. We therefore elected to subject bases belonging only the 5′-UTR to phylogenetic analysis. Coding sequences inclusive of the putative ATG start codon were excluded from our phylogenetic analysis.

The bootstrap proportion derived from the consensus trees should be interpreted carefully [Felsenstein, 1988]. They should not be perceived as the confidence intervals of statistical analyses. A conservative approach would be simply to use the values as a rough guide of the comparative support for the various groups of interest present in the same tree [Hillis and Bull, 1993]. Felsenstein [1993] has also suggested that (1-P), where P is the bootstrap proportion, may be used as a conservative assessment of the probability of getting that much evidence favoring the group if it was not present. Hence, two different methods of phylogenetic analysis was used in this study. Although both parsimony and the neighbor-joining method were expected to yield the correct general topology, neighbor-joining may not give the minimum evolution tree [Hillis et al., 1992]. Different phylogenetic methods have different assumptions and the methods become inconsistent for some trees when these assumptions are violated [Felsenstein, 1988; Hillis et al., 1994].

The bootstrap values obtained in this study corroborate the findings of Muerhoff et al. [1997]. There appears to be relatively strong support for the existence of three major groups of GBV-C/HGV isolates, namely, groups 1, 2, and 3, irrespective of the length of sequence analyzed or the phylogenetic method used. We also confirm that the analysis of longer stretches of the 5′-UTR generally results in increased level of bootstrap support for the grouping. Furthermore, we confirm that group 2 can be subdivided into subgroups 2a and 2b. The findings of Kondo et al., [1997] contradict this latter point. However, if group 2 is not to be further subdivided, the group 2 isolates in this study should have been found distributed across the entire group 2 monophyletic branch, albeit in small clusters of comparative similarity. Instead, we find that 100% of our group 2 isolates are characterized as 2a and none as 2b. Moreover, it appears that group 2a is the more widely distributed of the two subgroups. It has been reported in Japan, China, Vietnam, as well as Argentina, the United States, and Europe [Muerhoff et al., 1997; Kakumu et al., 1998]. In contrast, group 2b has hitherto been reported only in the United States and Europe. Unlike the initial subdivision of group 1 into 1a and 1b, which has since been withdrawn [Muerhoff et al., 1997], the distinction between group 2a and group 2b seems to be preserved, as shown by our data.

Phylogenetic analyses of GBV-C/HGV isolates from several Asian countries, including Bangladesh, China, Japan, Korea, Mongolia, and Vietnam, have shown that both group 2 and 3 isolates can be found in this part of the world [Katayama et al., 1997; Kondo et al., 1997; Mukaide et al., 1997; Ping et al., 1997; Wu et al., 1997; Kakamu et al., 1998]. In this study, we found only 45% of our isolates belong to the Asian group 3 strain. Another 50% of our isolates segregated with group 2a. These isolates were found among both the Chinese and Malay patients in our sample, whereas group 3 isolates all came from Chinese patients. However, this relatively small sample size does not permit us to make any firm statistical inference from this racial distribution data.

We also report the occurrence of a single group 1-like isolate, SG57. A previous study of 11 GBV-C/HGV isolates from southern China noted, in addition to group 2 and 3 isolates, the occurrence of a single isolate that segregated with the GBV-C group 1 prototype, U36380 [Wu et al., 1997]. It is interesting to note that SG57 was isolated from a Malay individual. An alignment of SG57 with the prototype GBV-C isolate (U36380) and 19 other group 1 isolates from Muerhoff et al. [1996] (data not shown) produced 504 nucleotides for analysis. It revealed 21 point mutations in SG57 that were not present in any of the other group 1 reference isolates. Smith et al. [1997] suggested that the 5′-UTR from nucleotides −490 to −235 might be optimal for analyzing the grouping of a GBV-C/HGV isolate. In our isolates, this would have excluded regions with group-specific polymorphisms at nucleotides −179, −144,

–139, and from nucleotides –124 to –121. In contrast, an analysis of the region stretching from nucleotides –482 to –103 would have incorporated all these additional motifs as well.

In conclusion, our data show that isolates belonging to all three main groups of GBV-C/HGV can be detected in the Southeast Asian region, with groups 2a and 3 being dominant. Furthermore, the occurrence of a group 1-like GBV-C/HGV isolate suggests that the spread of GBV-C/HGV from other parts of the world is slow but may increase in the future. Intriguingly, GBV-C/HGV isolates from Nicaragua have been grouped with isolated from Asia, and the possibility that GBV-C/HGV was introduced into this Central American population by the Amerindian migration from Asia did not go unnoticed by the author [Gonzalez-Perez et al., 1997]. Evidence documenting the modes of transmission and clinical significance of GBV-C/HGV are accumulating [Mushahwar and Zukerman, 1998], and hence studies of the diversity and infection rates of GBV-C/HGV will be important in understanding the functioning of this virus.

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